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Preclinical Assessment of Efficacy of Radiation Dose Painting Based on Intratumoral FDG-PET Uptake

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Abstract

Purpose: We tested therapeutic efficacy of two dose painting strategies of applying higher radiation dose to tumor subvolumes with high FDG uptake (biologic target volume, BTV): dose escalation and dose redistribution. We also investigated whether tumor response was determined by the highest dose in BTV or the lowest dose in gross tumor volume (GTV).

Experimental Design: FDG uptake was evaluated in rat rhabdomyosarcomas prior to irradiation. BTV was defined as 30% of GTV with the highest (BTV_{hot}) or lowest (BTV_{cold}) uptake. To test efficacy of dose escalation, tumor response (time to reach two times starting tumor volume, TGT_{V2}) to Hot Boost irradiation (40% higher dose to BTV_{hot}) was compared with Cold Boost (40% higher dose to BTV_{cold}), while mean dose to GTV remained 12 Gy. To test efficacy of dose redistribution, TGT_{V2} after Hot Boost was compared with uniform irradiation with the same mean dose (8 or 12 Gy).

Results: TGT_{V2} after 12 Gy delivered heterogeneously (Hot and Cold Boost) or uniformly were not significantly different: 20.2, 19.5, and 20.6 days, respectively. Dose redistribution (Hot Boost) with 8 Gy resulted in faster tumor regrowth as compared with uniform irradiation (13.3 vs. 17.1 days; $P = 0.026$). Further increase in dose gradient to 60% led to a more pronounced decrease in TGT_{V2} (10.9 days; $P < 0.0001$).

Conclusions: Dose escalation effect was independent of FDG uptake in target tumor volume, while dose redistribution was detrimental in this tumor model for dose levels applied here. Our data are consistent with the hypothesis that tumor response depends on the minimum intratumoral dose. *Clin Cancer Res; 21(24); 5511–8. ©2015 AACR.*

Introduction

Noninvasive functional or molecular imaging techniques such as positron emission tomography (PET) allow detection of spatial distribution of biologic phenotypes within a tumor, which might serve as surrogates of radioresistance. It has been hypothesized that radiation dose painting, that is, the prescription of a nonuniform radiation dose distribution to the gross tumor volume (GTV), based on the image-guided identification of potentially radioresistant biologic target volumes (BTV) within the GTV, may improve radiotherapy outcome (1–3).

Regions with high ^{18}F-fluorodeoxyglucose (FDG) uptake within the metabolically active tumor areas are attractive targets for subvolume boosting, that is, while tumor subvolumes with elevated FDG avidity are irradiated with higher doses, reduced doses (redistribution approach) or standard curative doses (dose escalation approach) are delivered to the rest of the tumor volume with no or lower FDG uptake. The biologic rationale for this approach is that FDG uptake is heterogeneous within a tumor; it reflects tumor areas with high cell density, which are highly metabolically active, and may identify the regions of radioresistant tumor cells owing to hypoxia or other mechanisms of radioresistance (4–8). The hypothesis of a more radioresistant tumor phenotype within high FDG uptake regions is also supported by the observation that (i) FDG uptake before or early during treatment is an independent prognostic factor for the outcome of (chemo-) radiotherapy in various tumor entities (9–11); (ii) regions of high FDG uptake remain stable during radiotherapy (12), and (iii) the residual metabolically active regions and local recurrences after radiotherapy remain or appear in the high pretreatment FDG uptake areas within the irradiated target volume (13–16).

These clinical data support the use of FDG PET imaging in target definition for dose painting. Numerous clinical studies in several cancers have demonstrated the feasibility to selectively escalate the dose to the tumor regions with increased FDG uptake (17–25). Yet, evidence for the therapeutic benefit of dose painting strategies from clinical trials is

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Translational Relevance

Clinical studies have demonstrated (i) that tumor relapse after irradiation occurs preferentially in tumor areas with high ^{18}F-fluorodeoxyglucose (FDG) uptake and (ii) the feasibility to selectively escalate radiation dose to the potentially radioresistant tumor subvolumes (dose painting) with increased FDG uptake without exceeding normal tissue tolerance. Yet, evidence proving the therapeutic gain of dose painting strategies from clinical trials is still lacking. Therefore, in this preclinical study in rat rhabdomyosarcoma, we tested therapeutic efficacy of two dose painting strategies to boost tumor subvolumes with high FDG uptake: (i) targeted dose escalation and (ii) dose redistribution. Although dose escalation to high FDG uptake was not superior to the same dose increase in low FDG uptake subvolumes, dose redistribution was even detrimental. Our data are consistent with the hypothesis that tumor response is dependent on the minimum intratumoral dose. Our findings may provide important information for the design of clinical trials.

still lacking. In this preclinical study in rats bearing syngeneic subcutaneous rhabdomyosarcoma tumors, we aimed to evaluate the therapeutic efficacy of two dose painting strategies to boost tumor subvolumes with high FDG uptake: (i) targeted dose escalation and (ii) dose redistribution. Our hypothesis that FDG high uptake regions are more radioresistant in rhabdomyosarcoma model is supported by preliminary observation demonstrating large overlap between high FDG uptake regions and high uptake of HX4 hypoxia PET tracer (Supplementary Fig. S1; refs. 26, 27), supporting accumulation of FDG in radioresistant hypoxic tumor regions. The feasibility of this novel radiation treatment approach in rats using state-of-the-art clinical imaging and irradiation devices, including 3D portal dosimetry, has been reported previously (28). To test the effect of dose escalation on tumor growth, 40% higher dose was delivered to tumor subvolumes with high FDG uptake than to the rest of the tumor (Hot Boost). Tumor response to this treatment was compared with the tumor response to the control treatment, that is, the same 40% increase in dose to nontarget tumor subvolumes with low FDG uptake (Cold Boost). According to our hypothesis, boosting FDG high uptake subvolumes should be more effective as compared with boosting FDG low uptake subvolumes, assuming that regions with high FDG uptake include the majority of radioresistant tumor cells. To test the effect of dose redistribution strategy, a 40% or a 60% higher dose was delivered to tumor subvolumes with high FDG uptake, while the rest of the tumor was irradiated with a lower dose. The effect of this redistribution treatment on tumor regrowth was compared with that of uniform irradiation that served as a control. Importantly, mean dose to GTV in experimental and control arms was kept the same. In this complex study, we tested isotoxic approach that is also used in ongoing clinical trials (NCT01024829, NCT01504815), where patients are randomized between dose escalation of the entire primary tumor or to the high FDG uptake regions inside the primary tumor (20). The preclinical model used in this study will be of great value to test the numerous different dose painting approaches that cannot be tested in the clinic.

Materials and Methods

Animals and tumor model

Animal studies were conducted in accordance with guidelines and approval of the Animal Ethical Committee of the University of Maastricht (Maastricht, the Netherlands). The experiments were performed using adult male WAG/Rij rats (weight $\geq$ 250 g) and previously well-characterized syngeneic rhabdomyosarcoma R1 tumor model (kindly provided by W. Landuyt, Experimental Radiotherapy, Katholieke Universiteit, Leuven, Belgium; refs. 29–33). For the experiments, tumor pieces (ca. 1 mm^3) were implanted subcutaneously (s.c.) in the left flank of anesthetized animals. Tumor pieces were used in the sequential series for at most 10 to 12 transplantations; thereafter transplantation was restarted from the stock of frozen cells.

Experimental design and tumor response evaluation

The study design is outlined in Supplementary Fig. S2. When tumors reached an average volume of 8.1 cm^3 [standard deviation (SD), 2.6 cm^3], rats were subjected to computed tomography (CT) or PET/CT imaging. Rhabdomyosarcoma tumors at this size have an average necrotic fraction of 16% (range, 5%–35%; $n = 9$; unpublished data), which is in the range of human tumor xenografts (34). Several hours after PET/CT imaging, the tumors were either left untreated or a mean single dose to the GTV of 8 or 12 Gy was delivered as uniform or heterogeneous irradiation. The two dose levels applied in this study have been selected on the basis of the previous results, demonstrating significant growth delay after uniform irradiation with these doses (33, 35). In all dose groups with heterogeneous irradiation, the BTV was defined to represent 30% of the GTV. The radiation doses are specified in Table 1.

The dose escalation strategy was tested for the mean dose to the GTV of 12 Gy. The following radiation treatments were compared:

1) Hot Boost 40%: 40% higher dose to BTV_{hot} [i.e., 30% of the GTV with the highest standardized uptake value (SUV)] than to the rest of the tumor (GTV-BTV).
2) Cold Boost 40%: 40% higher dose to BTV_{cold} (i.e., 30% of the GTV with the lowest SUV) than to the rest of the tumor (control).

The dose redistribution strategy was tested for the mean dose to the GTV of 8 or 12 Gy, which was kept constant for all treatment arms:

1) Hot Boost 40%.
2) Hot Boost 60%: 60% higher dose to BTV_{hot} than to the rest of the tumor (only for 8 Gy mean dose).
3) Uniform irradiation (control).

In addition, the groups of tumors were uniformly irradiated with 4, 6, or 15 Gy to obtain dose–response relationships. The response of tumors to various radiation treatments was evaluated by a tumor growth delay assay. Tumors were measured three times per week using a Vernier caliper, and volumes were

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Table 1. Constraints for the target structures and organs at risk for uniform irradiation, heterogeneous boost of hot or cold FDG subvolumes with 40% or 60% higher dose for the mean dose of 12 and/or 8 Gy

Constraints	Uniform	Heterogeneous boost (40% dose gradient)	Heterogeneous boost (60% dose gradient)
Mean GTV dose	12 (8)	12 (8)	8
Min GTV dose ($D_{99\%}$)	≥10.8 (7.2)	—	—
Max GTV dose	≤13.2 (8.8)	—	—
Mean BTV dose	—	15 (10)	10.85
Min BTV dose ($D_{99\%}$)	—	13.5 (9)	8.7
Max BTV dose	—	17.25 (11.5)	12.5
Mean (GTV-BTV) dose	—	10.7 (7.1)	6.8
Min (GTV-BTV) dose ($D_{99\%}$)	—	9.6 (6.4)	5.44
GI V_{8Gy}	≤5%	≤5%	≤5%
Max spinal cord dose	≤7	≤7	≤7

NOTE: All doses are reported in units of Gy.

Abbreviations: GI, gastrointestinal tract; $D_{99\%}$, 99% of the volume receives dose ≥$D_{99\%}$; V_{8Gy}, the maximal fractional volume of the organ receiving dose ≥ 8 Gy.

calculated as $(a \times b \times c) \times \pi/6$, where a, b, and c are the orthogonal dimensions corrected for the thickness of skin. Animals were observed until the tumor volume exceeded 25 cm^3, until death, or until the animal appeared to suffer. Tumor response was quantified as the time required to reach two times the starting tumor volume as determined on the day of PET/CT scan (TGT_{V2}).

PET/CT data acquisition, image segmentation, treatment planning, and irradiation

PET/CT images were acquired using a clinical PET/CT scanner (Biograph 40; Siemens Healthcare). Two hours prior to PET/CT imaging, FDG [19.9 (2.8) MBq] was injected intravenously (i.v.) while animals were sedated with a mixture of ketamine/xylazine [100 and 10 mg/kg, respectively, intraperitoneal (i.p.)]. Several minutes prior to scanning (20-minute duration), alignment was performed for each anesthetized (ketamine/xylazine) animal with the laser guides; cross-hairs and lines were drawn with skin-ink on previously shaved body areas for subsequent accurate repositioning of the rats for irradiation. The tumors were covered with 1-cm thick super stuff bolus (Radiation Product Design, Inc.).

PET/CT images were acquired with an axial field of view (FoV) of 162 mm and a spatial resolution of 5.3-mm FWHM at the center of the FoV. The PET data were corrected for photon attenuation using the acquired CT images. Correction for scatter (3D), random counts, dead time, and decay of injected radionuclides was also applied. First, a topogram was acquired followed by a whole-body CT scan using a 1-mm reconstructed slice thickness and a pitch of 0.8. Finally, for PET imaging a 20-minute emission scan in list mode in one bed position was acquired and reconstructed as 4×5 minutes. FDG uptake was quantified by maximal SUV (SUV_{max}) as maximal FDG activity in GTV corrected for the decay, injected dose, and body weight.

CT images were directly imported into Eclipse treatment planning system (TPS; v11, Varian Medical Systems) for uniform irradiation treatment planning. To plan heterogeneous dose distribution irradiation, PET/CT images were first imported into Imalytics 3.0 (Philips Technologies GmbH) for tumor segmentation and BTV determination. The GTV was manually delineated on CT scans. PET images were segmented to obtain BTV_{hot} and BTV_{cold} within GTV (*vide supra*). Next, the GTV and BTV contours were imported into the Eclipse TPS. Finally, the animal body and spinal cord in the treatment field were segmented automatically, whereas the abdominal region containing the gastrointestinal tract was manually delineated. For uniform irradiation, Rapid Arc VMAT treatment plans were created using a single full arc, while for heterogeneous plans two full arcs were required for optimal dose distribution. Dose calculations were performed with the Eclipse AcurosXB 10.0 algorithm (Varian Medical Systems) using the smallest grid size of 0.1 cm. The dose constraints for the target structures and organs at risk are summarized in Table 1. Dose homogeneity for the GTV in the case of uniform irradiation is defined in such a way that 99% of the volume needs to receive 90% to 110% of the prescribed dose, while 90% to 115% (80%–115% for 60% dose gradient) of the prescribed dose has to be delivered to the BTV (20).

Several hours after PET/CT scan, anesthetized (sodium pentobarbital, 60 mg/kg, i.p.) animals were repositioned on the couch of a TrueBeam STx High-Definition 120 Multileaf Collimator linac (Varian Medical Systems). A cone beam CT (CBCT) scan was then acquired with the on-board imager (100 kVp, 73.2 mAs) and matching of the CBCT to the planning CT scans was performed prior to irradiation. Animals were anesthetized for ca. 15 minutes before irradiation (duration maximum 14 minutes) could be performed. Some animals (3 of 51) had to be repositioned up to three times with the subsequent CBCT scan to achieve acceptable repositioning. After treatment, the dose metrics to the target structures were recalculated on the basis of CBCT images as described previously (28).

Metabolic response assessment

FDG PET/CT imaging of tumors irradiated with a mean dose of 12 Gy was performed 7 days after treatment as described above. The pre- and posttreatment CT and PET scans were delineated and segmented in Imalytics 3.0 (Philips Technologies GmbH). The location and volume of the FDG uptake areas within the GTV were quantified using the thresholds 10%, 20%, 30%, 40%, and 50% of the GTV with the highest SUV on both pre- and posttreatment PET scans. CT images and PET contours were then imported into the SmartAdapt image registration application (v11, Varian Medical Systems). For most of the animals (8 of 12), the difference between pre- and posttreatment tumor volume was less than 20%. The maximum observed difference between pre- and posttreatment GTV was 41%. In SmartAdapt using an automatic rigid registration algorithm based on mutual information from CT scans, the CT images of the posttreatment scan were fused to the images of the pretreatment CT scan. If the automatic registration resulted in poor matching between the two scans, the images were manually registered on the basis of the tumor contours and anatomy of surrounding bony structures and soft tissue. Then, the PET contours on the posttreatment scan were propagated to the pretreatment scan and the Dice similarity coefficient (DSC) was calculated as $2\times[(V_{pre}\cap V_{post})/(V_{pre}+V_{post})]$, where V_{pre} and V_{post} are the volumes of the FDG-based segmentations on pre- and posttreatment PET scans.

Statistical analysis

GraphPad Prism software (version 5.00 for Windows, GraphPad Software) was used to perform statistical analyses. Mean values with SDs are reported. Mean values were compared using the independent sample t test. P values were adjusted for multiple comparisons using the Bonferroni correction when relevant. Linear regression analysis was used to test the correlations

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between various parameters. *P* values less or equal to 0.05 were considered as statistically significant.

Results

The FDG uptake as quantified by SUV_{max} was similar between the different experimental arms. The average SUV_{max} was 4.33 (1.57; $n = 42$). Across different experimental groups, an average BTV of 29.6% (2.3) was obtained. Small deviations from the desired BTV of 30% can be explained by slight volumetric changes occurring during transfer of the target structures between different software applications. Some examples of dose distributions for uniform irradiation, Hot Boost and Cold Boost heterogeneous irradiation are shown in Fig. 1. The planned doses (calculated on the basis of the CT image) and delivered doses (calculated on the basis of the CBCT image) to the target structures as well as dose volume histogram (DVH) metrics for mean dose 8 Gy are summarized in Supplementary Table S1. These data for 12 Gy have been reported previously (28). Visual rigorous inspection has identified four tumors for a new rigid registration to improve the match of CT and CBCT images and to calculate the delivered dose. Overall, in most of the cases (25 of 27) the discrepancy between planned and delivered doses and DVH metrics was less than 3% for all target structures. In the remaining two cases, the maximum difference was 4.8% for the GTV $D_{5\%}$, uniform

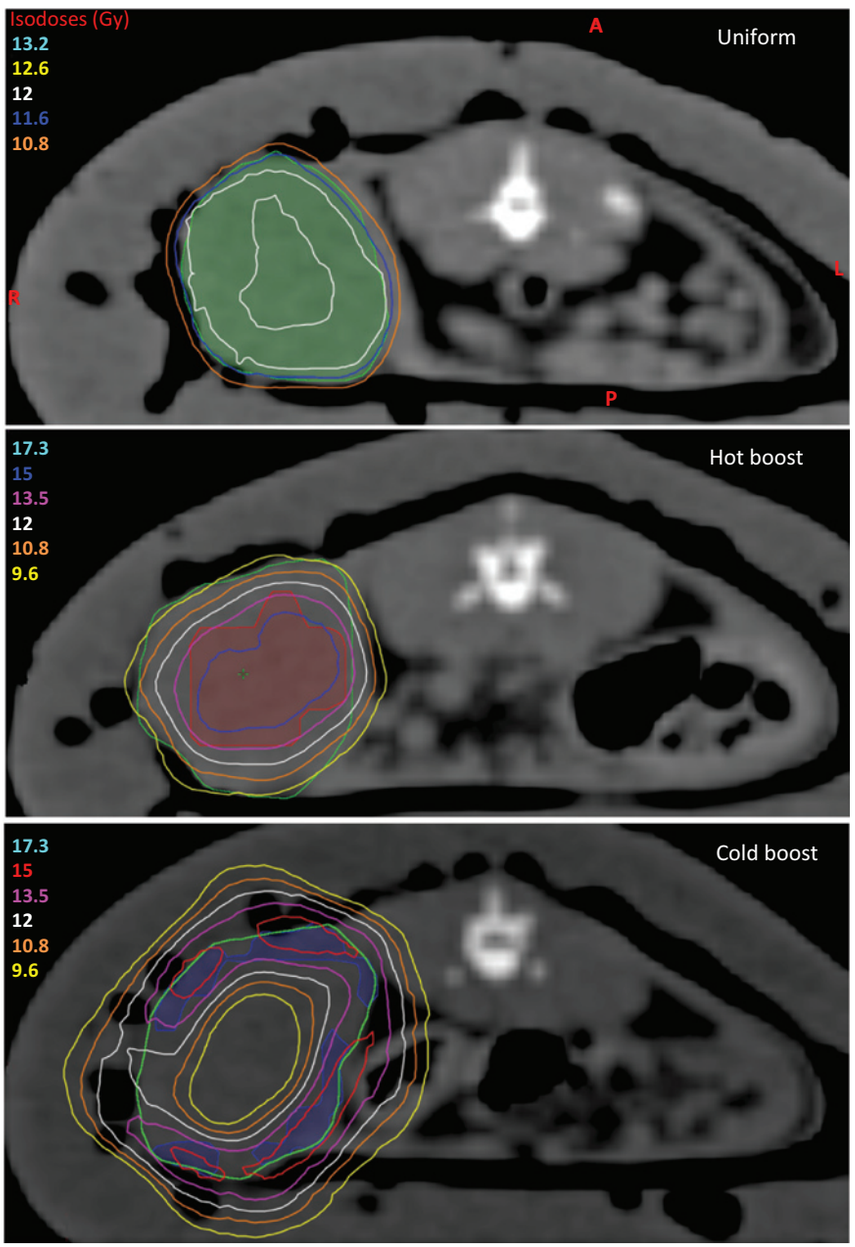

Figure 1.
Examples of treatment plans for uniform dose distribution, Hot Boost and Cold Boost heterogeneous irradiation. A mean dose of 12 Gy was prescribed to the GTV (green contour and shading) and 15 Gy to the BTV with the highest (hot, red contour and shading) or lowest (cold, blue contour and shading) FDG uptake.

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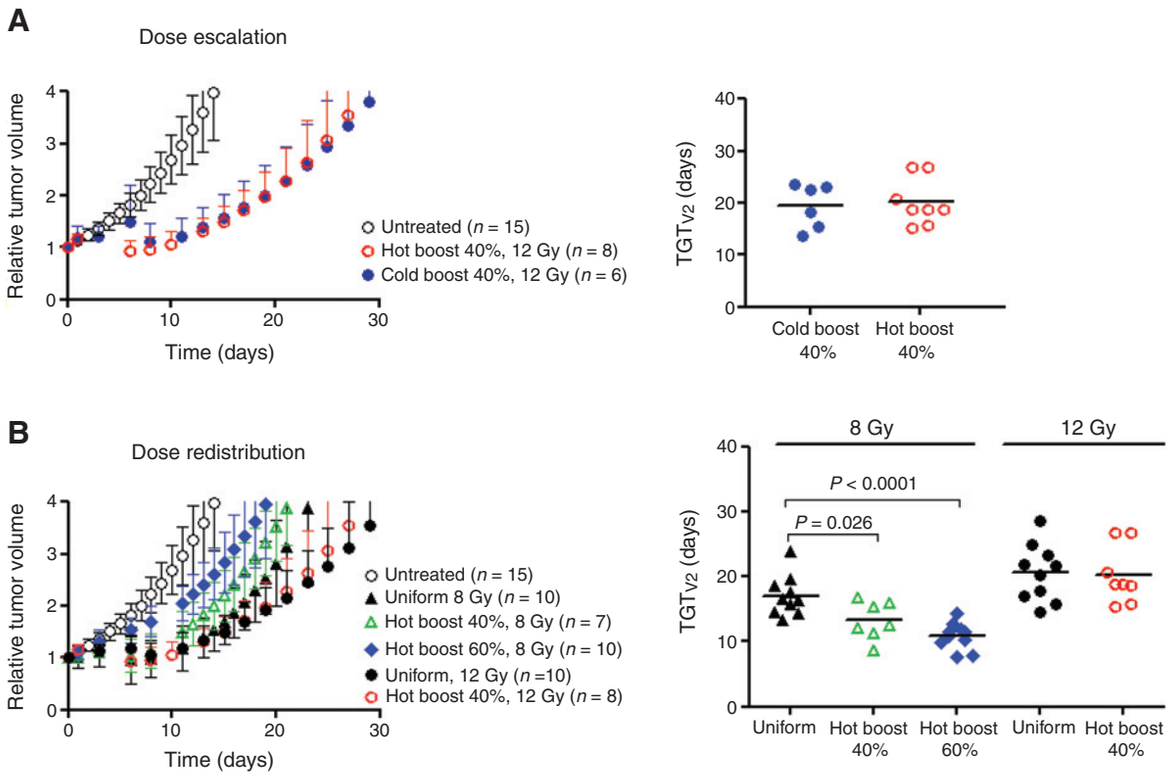

Figure 2.
Growth curves of untreated tumors and tumors irradiated uniformly or heterogeneously with a mean GTV dose of 12 or 8 Gy to test efficacy of (A) dose escalation and (B) dose redistribution approaches. Tumor response to radiation treatments was quantified as the time required to reach two times the starting tumor volume (TGT_{V2}). Each symbol represents a mean value; error bar, SD.

irradiation, indicating that the planned doses and dose homogeneity in both low- and high-dose regions could be accurately achieved. Radiation toxicity was not observed in any of the treatment groups, regardless of the radiation protocol.

Rhabdomyosarcoma tumor model demonstrated clear dose–response relationships, that is, TGT_{V2} significantly increases with increasing uniform radiation doses (Supplementary Fig. S3). Increasing dose by 40% to BTV_{hot} with the highest FDG uptake did not have a greater effect on tumor growth than the same dose increase to BTV_{cold} with the lowest uptake after irradiation with mean dose of 12 Gy (Fig. 2A), indicating that this dose-escalation strategy based on FDG uptake did not result in therapeutic benefit in a rat rhabdomyosarcoma tumor model. Efficacy of the dose redistribution approach has been tested for two dose levels by comparing the tumor response to Hot Boost irradiation with that to uniform irradiation, while keeping the mean dose to the GTV constant. Redistributing a mean dose of 12 Gy in a way that BTV_{hot} received a 40% higher dose than the remaining tumor volume (GTV-BTV) resulted in TGT_{V2} that was not significantly different from TGT_{V2} after uniform irradiation: 20.2 (4.4) vs. 20.6 (4.4) days (Fig. 2B). In contrast, redistributing a mean dose of 8 Gy in the same way resulted in significantly faster tumor regrowth as compared with uniform 8-Gy irradiation [13.3 (2.9) vs. 17.1 (3.1) days; $P = 0.026$; Fig. 2B]. Further increase of the dose gradient between BTV and (GTV-BTV) to 60% (Table 1) led to an even more pronounced decrease in TGT_{V2} [10.9 (2.1) days; $P < 0.0001$), that is, to a worsening of radiation response (Fig. 2B).

Metabolic response was assessed in tumors irradiated with a mean dose of 12 Gy delivered uniformly or as a Hot Boost 7 days after treatment. The overlap between residual and pretreatment high FDG uptake subvolumes as quantified by DSC showed pronounced variation (Fig. 3). In particular, the DSC for 30% of the total tumor volume with the highest SUV uptake varied between 0.37 and 0.73 for uniform irradiation and between 0.29 and 0.72 for the Hot Boost irradiation. As expected, DSC increased with increasing thresholds from 10% to 50% of the GTV with the highest FDG uptake (Fig. 3). The average DSCs were not significantly different between the radiation protocols for any of the subvolumes segmented on the basis of various thresholds on pre- and posttreatment FDG scans.

Discussion

This, to the knowledge of the authors, is the first study assessing the effect of radiation dose painting on tumor growth based on FDG uptake in rat tumors using two strategies of nonuniform dose distribution: dose escalation and dose redistribution. The technical feasibility to deliver nonuniform dose distributions to tumors in rats using state-of-the-art clinical imaging and irradiation devices has been reported previously in detail (28). In this study, boosting tumor subvolumes with high FDG uptake with a 40% higher radiation dose resulted in the same growth inhibition as the same dose escalation to the subvolumes with low FDG uptake. Furthermore, redistributing

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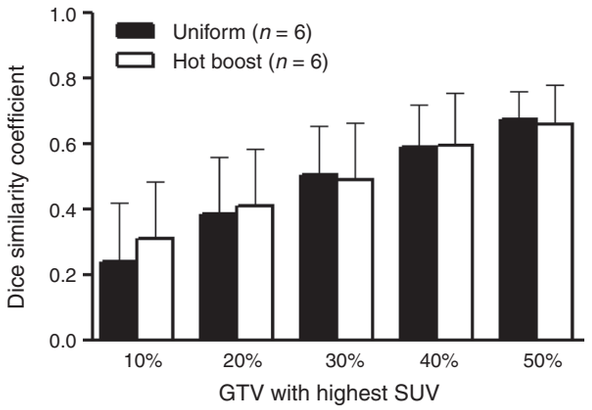

Figure 3.
The DSC representing spatial overlap of the pre- and posttreatment subvolumes ranging between 10% and 50% of GTV with the highest SUV for uniform and Hot Boost irradiation with mean GTV dose 12 Gy. Data, mean ± SD.

the mean dose 12 Gy in such a way that the tumor subvolumes with high FDG uptake received a 40% higher dose than the rest of the tumor volume did not result in therapeutic gain as compared with uniform irradiation with the same mean dose to the GTV. For the lower dose level of 8 Gy, the same dose redistribution by 40% resulted in faster tumor growth, and therefore worse radiation response, as compared with uniform irradiation. This negative effect on tumor growth was further enhanced if 60% higher dose was delivered to subvolumes with high FDG uptake, while dose to the rest of the tumor was further decreased maintaining the mean dose to the GTV at 8 Gy. Our findings in a rat rhabdomyosarcoma tumor model using two dose levels do not support the radiation dose boosting approach with nonuniform dose distributions based on FDG uptake. More importantly, a dose redistribution approach, that is, a decrease of dose to tumor subvolumes with low FDG uptake, while boosting the high FDG uptake subvolumes, may even be detrimental, if the former dose is lower than a standard curative dose in a clinical situation. Our data, therefore, support the hypothesis that tumor response depends on the minimum intratumoral dose.

The lack of effect of dose escalation to the tumor subvolumes with high FDG uptake on tumor growth was not expected because several clinical studies have shown that tumor relapse after irradiation occurs preferentially in tumor areas with high FDG uptake (13–15). Moreover, a number of correlative preclinical and clinical studies have shown that tumor areas with high FDG uptake are associated with decreased local tumor control and thereby with increased radioresistance (8–11). This association, however, was not confirmed in some clinical studies (36, 37). One might anticipate that in the rat rhabdomyosarcoma tumors investigated in this study tumor cells with high FDG uptake are not more radioresistant making dose-escalation strategy based on FDG uptake ineffective in this tumor model. One of the explanations for the differential radiosensitivity between low and high FDG uptake tumor regions may be predominant FDG accumulation in radioresistant hypoxic areas. Preclinical and clinical studies comparing spatial distribution of FDG and hypoxia tracers, however, reported diverse results showing complete and partial overlap or even clear spatial discordance in uptake of two tracers (1, 6–8, 23, 38, 39). The spatial correlation of FDG and a hypoxia tracer has not been tested systematically in the rat rhabdomyosarcoma model investigated here, but preliminary observations do not exclude at least a partial overlap between high FDG uptake regions and high uptake of HX4 hypoxia PET tracer (Supplementary Fig. S1; refs. 26, 27), supporting accumulation of FDG in hypoxic tumor regions. In addition, metabolic response data in this study showed diverse patterns of correspondence between post- and pretreatment metabolically active subvolumes from very poor to a large overlap. A time point 7 days after irradiation with 12 Gy prior to tumor regrowth (Fig. 2) was chosen for evaluation. The value of DSC of pre- and posttreatment high FDG-uptake subvolumes was independent of radiation protocol, while it was hypothesized that their overlap would be higher after uniform irradiation as compared with radiation Hot Boost, owing to the higher cell kill by the higher dose in the BTV. Taken together, the data suggest that regions of high FDG uptake are not more radioresistant in rat rhabdomyosarcomas and may not contribute to the tumor regrowth after irradiation. Because tumor response improves with increasing uniform radiation doses to the entire tumor (Supplementary Fig. S3), supporting efficacy of dose escalation, the present data stresses the importance of the development of tools to accurately identify target tumor volume for dose painting. Hypothetically, only patients in whom tracer uptake identifies location of radioresistant cells and of local recurrences, for examples, subvolumes with high density of cancer stem cells (25, 40), may benefit from targeted dose escalation. Because spatial discordance between FDG and a hypoxia tracer uptake may exist, the combination of both tracers might be a powerful tool to determine high-risk tumor subvolumes (7).

In contrast to our findings, a preclinical study in human head and neck squamous cell carcinoma xenografts of Schuetze and colleagues (41) have shown that 40% increase of radiation dose from 25 to 35 Gy had a greater effect on radiation response in tumors with high pretherapeutic FDG uptake than in tumors with low FDG uptake. This preclinical investigation, however, cannot be directly compared with this study because uniform irradiation was delivered to the tumors stratified by median SUV_{max} to low or high FDG uptake tumors assuming that each tumor represents a subvolume of a tumor in a single patient, whereas in this study, the nonuniform dose distributions were applied on the basis of intratumoral FDG uptake. Here, we used lower radiation doses of 8 and 12 Gy that have been shown previously to induce significant growth delay in rhabdomyosarcoma tumor model when delivered uniformly (33, 35). Moreover, TGT_{V2} increases with increasing uniform radiation doses (Supplementary Fig. S3). Therefore, based on the tumor response to uniform irradiation, it is expected that 4-Gy dose increment, for example, in Hot Boost radiation group (mean dose 12 Gy: 10.7 Gy in (GTV-BTV) vs. 15 Gy in BTV), would result in increase of TGT_{V2} under the assumption that tumor regrowth predominantly depends on tumor cells with high FDG uptake, thereby supporting the choice of radiation doses used in the present growth delay assay. Another important difference is that in the study by Schuetze and colleagues, an endpoint of local tumor control was evaluated as opposed to TGT_{V2} in the present growth delay assay. Nevertheless, radiation growth delay is a valid assay to obtain indications on the effect of novel approaches tested here with further validation in local control experiments, which is supported by the

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observations that (i) growth delay reflects radiation-induced cell kill, and (ii) growth delay correlates with local tumor control after irradiation (42).

Remarkably, we demonstrate in this study that a decrease of radiation dose to the tumor subvolumes with low FDG uptake, while increasing the dose to high FDG uptake subvolumes in dose redistribution approach is detrimental for some dose levels. This negative effect is more pronounced the greater the dose difference between the low and high FDG uptake regions, for the same mean radiation dose. The importance of the dose delivered to the low uptake tumor regions (GTV-BTV) is also supported by a just significant positive correlation between minimum (GTV-BTV) dose $D_{95\%}$ (Supplementary Table S1) and radiation response after 8-Gy 40% dose gradient ($R^2 = 0.56$; $P = 0.05$), that is, higher minimum dose $D_{95\%}$ results in a greater tumor growth delay. Notably, this correlation was insignificant for the BTV target structure. Overall, based on these results, the dose to the low FDG uptake subvolumes should not be lower than a standard curative dose in order to avoid undertreating the patients.

Several other assumptions and limitations have to be discussed. First, BTV was defined to represent 30% of the entire tumor volume with the highest FDG uptake, whereas in clinical studies BTV is defined as a tumor subvolume with SUV values above a certain threshold, for example, 50% SUV_{max} (20, 37, 43). However, a standard method for the determination of an optimal threshold for clinical use has not been established yet. In clinical studies, BTVs are determined in different tumors in different patients who have different genetic backgrounds, whereas in this preclinical study, we assumed that tumors with identical genetic background and in the same host have a similar proportion of radioresistant cells. This assumption is also supported by similar FDG uptake across rhabdomyosarcoma tumors investigated here. Moreover, the fractional BTV of 30% in this study corresponds well with the average highly metabolic fractional volume in clinical studies, including ongoing clinical trial (NCT01024829) testing the same isotoxic dose painting approach in patients (7, 20). Importantly, constant BTV allows to include appropriate controls to maintain mean dose to the entire tumor on the same level. Second, in this proof-of-principle study single dose regimens, although at two dose levels were tested, which limits translation of the results into the clinical situation. Third, as only one tumor model was studied confirmatory investigations using further tumor models are warranted.

In conclusion, tumor response to dose escalation was independent of whether radiation dose was increased to tumor subvolumes with high or low FDG uptake using nonuniform radiation protocols in rat rhabdomyosarcomas. This approach is therefore not recommended for testing in clinical trials unless it has been demonstrated that BTV areas are stable and are more radioresistant. Decreasing radiation dose to tumor subvolumes with low FDG uptake, while boosting high FDG uptake regions using dose redistribution approach may be detrimental. Our data are consistent with the hypothesis that tumor response depends on the minimum intratumoral dose.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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